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A

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. § 1.53 (b))

Attorney Docket No. LEX-0042-USA

First Inventor or Application Identifier Gregory Donoho et al.

Title Novel Human Protease Inhibitor-Like Proteins and Polynucleotides Encoding the Same

Express Mail label No. EL584856694US

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents

1 *Fee Transmittal Form (e.g., PTO/SB/17)
(Submit an original and a duplicate for fee processing)

2 Specification [Total Pages] 25

- Descriptive title of the Invention
- Cross References to Related Applications
- Statement Regarding Fed sponsored R & D
- Reference to Microfiche Appendix
- Background of the Invention
- Brief Summary of the Invention
- Brief Description of the drawings (if filed)
- Detailed Description
- Claim(s)
- Abstract of the disclosure

3 Drawing(s)(35 U.S.C.113) [Total Sheets] 1

4. Oath or Declaration [Total] 1

a. Newly unexecuted (original or copy)

b. Copy from a prior application (37 C.F.R. § 1.63(d))
(for continuation/divisional with Box 16 completed)

i. DELETION OF INVENTOR(S)
Signed statement attached deleting
inventor(s) named in the prior application,
see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b).

NOTE FOR ITEMS 1 & 13: IN ORDER TO BE ENTITLED TO PAY SMALL ENTITY FEES, A SMALL ENTITY STATEMENT IS REQUIRED (37 C.F.R. § 127), EXCEPT IF ONE FILED IN A PRIOR APPLICATION IS RELIED UPON (37 C.F.R. §128).

5 Microfiche Computer Program (Appendix)

6. Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)

- a. Computer Readable Copy
- b. Paper Copy (identical to computer copy)
- c. Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

- 7 Assignment Papers (cover sheet & document(s))
- 8 37 C.F.R. §3.73(b) Statement (when there is an assignee) Power of Attorney
- 9. English Translation Document (if applicable)
- 10. Information Disclosure Statement (IDS)/PTO-1449 Copies of IDS Citations
- 11. Preliminary Amendment
- 12. Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)
- 13. *Small Entity Statement(s) Statement filed in prior application, Status still proper and desired (PTO/B-09-12)
- 14. Certified Copy of Priority Document(s)
(if foreign priority is claimed)
- 15. Other:

16. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in a preliminary amendment:

Continuation Divisional Continuation-in-part (CIP) of prior application No: _____

Prior application information: Examiner _____ Group/Art Unit: _____

For CONTINUATION or DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 4b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference.

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(37 CFR 1.9(f) & 1.27(c)) -- SMALL BUSINESS CONCERN**

 Docket Number (Optional)
LEX-0042-USA
Applicant, Patentee, or Identifier: Gregory Donoho et al.

Application or Patent No.: _____

Filed or Issued: September 22, 2000Title: Novel Human Protease Inhibitor-Like Proteins and Polynucleotides Encoding the Same

I hereby state that I am

the owner of the small business concern identified below:
 an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF SMALL BUSINESS CONCERN Lexicon Genetics IncorporatedADDRESS OF SMALL BUSINESS CONCERN 4000 Research Forest Drive, The Woodlands, TX 77381

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NAME OF PERSON SIGNING Lance K. IshimotoTITLE OF PERSON IF OTHER THAN OWNER Vice President - Intellectual PropertyADDRESS OF PERSON SIGNING 4000 Research Forest Drive, The Woodlands, TX 77381SIGNATURE DATE September 22, 2000Reg. No. 41,866

NOVEL HUMAN PROTEASE INHIBITOR-LIKE PROTEINS AND POLYNUCLEOTIDES
ENCODING THE SAME

1. INTRODUCTION

5 The present application claims priority to U. S. Provisional Application Number 60/156,101 which was filed September 24, 1999 which is herein incorporated by reference in its entirety.

The present invention relates to the discovery, identification, and characterization of novel human 10 polynucleotides encoding proteins that share sequence similarity with mammalian trypsin inhibitors. The invention encompasses the described polynucleotides, host cell expression systems, the encoded proteins, fusion proteins, polypeptides and peptides, antibodies to the encoded proteins and peptides, and genetically 15 engineered animals that either lack or over express the disclosed genes, antagonists and agonists of the proteins, and other compounds that modulate the expression or activity of the proteins encoded by the disclosed genes that can be used for diagnosis, drug screening, clinical trial monitoring, the treatment of 20 physiological disorders, or otherwise contributing to the quality of life.

2. BACKGROUND OF THE INVENTION

Proteases are enzymes that mediate the proteolytic cleavage of polypeptide sequences. Conversely, protease inhibitors prevent 25 or hinder proteolytic activity. Given the importance of proteolysis in a wide variety of cellular functions and disease, protease inhibitors have been demonstrated to be involved in, *inter alia*, regulating development, modulating cellular processes, and preventing infectious, and particularly viral, disease.

30 3. SUMMARY OF THE INVENTION

The present invention relates to the discovery, identification, and characterization of nucleotides that encode

novel human proteins, and the corresponding amino acid sequences of these proteins. The novel human proteins (NHPs) described for the first time herein share structural similarity with animal trypsin inhibitor proteins. As such, the novel genes represent a 5 new class of proteins with a range of homologues and orthologs that transcend phyla and a range of species.

The novel human nucleic acid sequences described herein, encode proteins/open reading frames (ORFs) of 497 amino acids in length (see SEQ ID NO: 2).

10 The invention also encompasses agonists and antagonists of the described NHPs, including small molecules, large molecules, mutant NHPs, or portions thereof that compete with native NHP, peptides, and antibodies, as well as nucleotide sequences that can be used to inhibit the expression of the described NHPs (e.g., 15 antisense and ribozyme molecules, and gene or regulatory sequence replacement constructs) or to enhance the expression of the described NHP genes (e.g., expression constructs that place the described gene under the control of a strong promoter system), and transgenic animals that express a NHP transgene, or "knock-outs" 20 (which can be conditional) that do not express a functional NHP.

Further, the present invention also relates to processes of identifying compounds that modulate, *i.e.*, act as agonists or antagonists, of NHP expression and/or NHP product activity that utilize purified preparations of the described NHPs and/or NHP 25 product, or cells expressing the same. Such compounds can be used as therapeutic agents for the treatment of any of a wide variety of symptoms associated with biological disorders or imbalances.

4. DESCRIPTION OF THE SEQUENCE LISTING AND FIGURES

30 The Sequence Listing provides the sequences of a trypsin inhibitor-like ORF that encodes the described NHP amino acid sequences.

5. DETAILED DESCRIPTION OF THE INVENTION

The NHPs, described for the first time herein, are novel proteins that are expressed in, *inter alia*, human cell lines, and human prostate, fetal brain, cerebellum, spinal cord, thymus, 5 spleen, lymph node, bone marrow, trachea, lung, kidney, fetal liver, thyroid, adrenal gland, stomach, small intestine, colon, muscle, heart, uterus, placenta, mammary gland, and testis cells. The described sequences were compiled from gene trapped cDNAs and clones isolated from a human testis cDNA library, and a human 10 placenta cDNA (Edge Biosystems, Gaithersburg, MD). The present invention encompasses the nucleotides presented in the Sequence Listing, host cells expressing such nucleotides, the expression products of such nucleotides, and: (a) nucleotides that encode mammalian homologs of the described genes, including the 15 specifically described NHPs, and the NHP products; (b) nucleotides that encode one or more portions of the NHPs that correspond to functional domains, and the polypeptide products specified by such nucleotide sequences, including but not limited to the novel regions of any active domain(s); (c) isolated nucleotides that 20 encode mutant versions, engineered or naturally occurring, of the described NHPs in which all or a part of at least one domain is deleted or altered, and the polypeptide products specified by such nucleotide sequences, including but not limited to soluble proteins and peptides in which all or a portion of the signal 25 sequence is deleted; (d) nucleotides that encode chimeric fusion proteins containing all or a portion of a coding region of an NHP, or one of its domains (e.g., a receptor binding domain, accessory protein/self-association domain, etc.) fused to another peptide or polypeptide; or (e) therapeutic or diagnostic derivatives of the 30 described polynucleotides such as oligonucleotides, antisense polynucleotides, ribozymes, dsRNA, or gene therapy constructs comprising a sequence first disclosed in the Sequence Listing.

As discussed above, the present invention includes: (a) the human DNA sequences presented in the Sequence Listing (and vectors comprising the same) and additionally contemplates any nucleotide sequence encoding a contiguous NHP open reading frame (ORF) that

5 hybridizes to a complement of a DNA sequence presented in the Sequence Listing under highly stringent conditions, *e.g.*, hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds., 1989, Current

10 Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encodes a functionally equivalent gene product.

Additionally contemplated are any nucleotide sequences that hybridize to the complement of the DNA sequence that encode and

15 express an amino acid sequence presented in the Sequence Listing under moderately stringent conditions, *e.g.*, washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989, *supra*), yet still encode a functionally equivalent NHP product. Functional equivalents of a NHP include naturally occurring NHPs present in

20 other species and mutant NHPs whether naturally occurring or engineered (by site directed mutagenesis, gene shuffling, directed evolution as described in, for example, U.S. Patent No. 5,837,458). The invention also includes degenerate nucleic acid variants of the disclosed NHP polynucleotide sequences.

25 Additionally contemplated are polynucleotides encoding NHP ORFs, or their functional equivalents, encoded by polynucleotide sequences that are about 99, 95, 90, or about 85 percent similar to corresponding regions of SEQ ID NO:1 (as measured by BLAST sequence comparison analysis using, for example, the GCG sequence

30 analysis package using standard default settings).

The invention also includes nucleic acid molecules, preferably DNA molecules, that hybridize to, and are therefore the complements of, the described NHP gene nucleotide sequences. Such

hybridization conditions may be highly stringent or less highly stringent, as described above. In instances where the nucleic acid molecules are deoxyoligonucleotides ("DNA oligos"), such molecules are generally about 16 to about 100 bases long, or about 5 20 to about 80, or about 34 to about 45 bases long, or any variation or combination of sizes represented therein that incorporate a contiguous region of sequence first disclosed in the Sequence Listing. Such oligonucleotides can be used in conjunction with the polymerase chain reaction (PCR) to screen 10 libraries, isolate clones, and prepare cloning and sequencing templates, etc..

Alternatively, such NHP oligonucleotides can be used as hybridization probes for screening libraries, and assessing gene expression patterns (particularly using a micro array or high- 15 throughput "chip" format). Additionally, a series of the described NHP oligonucleotide sequences, or the complements thereof, can be used to represent all or a portion of the described NHP sequences. The oligonucleotides, typically between about 16 to about 40 (or any whole number within the stated range) 20 nucleotides in length may partially overlap each other and/or the NHP sequence may be represented using oligonucleotides that do not overlap. Accordingly, the described NHP polynucleotide sequences shall typically comprise at least about two or three distinct oligonucleotide sequences of at least about 18, and preferably 25 about 25, nucleotides in length that are each first disclosed in the described Sequence Listing. Such oligonucleotide sequences may begin at any nucleotide present within a sequence in the Sequence Listing and proceed in either a sense (5'-to-3') orientation vis-a-vis the described sequence or in an antisense 30 orientation.

For oligonucleotide probes, highly stringent conditions may refer, e.g., to washing in 6xSSC/0.05% sodium pyrophosphate at 37°C (for 14-base oligos), 48°C (for 17-base oligos), 55°C (for

20-base oligos), and 60°C (for 23-base oligos). These nucleic acid molecules may encode or act as NHP gene antisense molecules, useful, for example, in NHP gene regulation (for and/or as antisense primers in amplification reactions of NHP gene nucleic acid sequences). With respect to NHP gene regulation, such techniques can be used to regulate biological functions. Further, such sequences may be used as part of ribozyme and/or triple helix sequences that are also useful for NHP gene regulation.

Inhibitory antisense or double stranded oligonucleotides can 10 additionally comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 15 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 20 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiacytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 25 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide can also comprise at least one modified sugar moiety selected from the group including but not 30 limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide will comprise at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate,

a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is
5 an α -anomeric oligonucleotide. An α -anomeric oligonucleotide
forms specific double-stranded hybrids with complementary RNA in
which, contrary to the usual β -units, the strands run parallel to
each other (Gautier *et al.*, 1987, *Nucl. Acids Res.* 15:6625-6641).
The oligonucleotide is a 2'-0-methylribonucleotide (Inoue *et al.*,
10 1987, *Nucl. Acids Res.* 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue *et al.*, 1987,
FEBS Lett. 215:327-330). Alternatively, double stranded RNA can
be used to disrupt the expression and function of a targeted NHP.

15 Oligonucleotides of the invention can be synthesized by
standard methods known in the art, e.g. by use of an automated DNA
synthesizer (such as are commercially available from Biosearch,
Applied Biosystems, etc.). As examples, phosphorothioate
oligonucleotides can be synthesized by the method of Stein *et al.*
20 (1988, *Nucl. Acids Res.* 16:3209), and methylphosphonate
oligonucleotides can be prepared by use of controlled pore glass
polymer supports (Sarin *et al.*, 1988, *Proc. Natl. Acad. Sci.*
U.S.A. 85:7448-7451), etc.

Low stringency conditions are well known to those of skill in
25 the art, and will vary predictably depending on the specific
organisms from which the library and the labeled sequences are
derived. For guidance regarding such conditions see, for example,
Sambrook *et al.*, 1989, *Molecular Cloning, A Laboratory Manual* (and
periodic updates thereof), Cold Springs Harbor Press, N.Y.; and
30 Ausubel *et al.*, 1989, *Current Protocols in Molecular Biology*,
Green Publishing Associates and Wiley Interscience, N.Y.

Alternatively, suitably labeled NHP nucleotide probes can be used to screen a human genomic library using appropriately stringent conditions or by PCR. The identification and characterization of human genomic clones is helpful for 5 identifying polymorphisms (including, but not limited to, nucleotide repeats, microsatellite alleles, single nucleotide polymorphisms, or coding single nucleotide polymorphisms), determining the genomic structure of a given locus/allele, and designing diagnostic tests. For example, sequences derived from 10 regions adjacent to the intron/exon boundaries of the human gene can be used to design primers for use in amplification assays to detect mutations within the exons, introns, splice sites (e.g., splice acceptor and/or donor sites), etc., that can be used in diagnostics and pharmacogenomics.

15 Further, a NHP gene homolog can be isolated from nucleic acid from an organism of interest by performing PCR using two degenerate or "wobble" oligonucleotide primer pools designed on the basis of amino acid sequences within the NHP products disclosed herein. The template for the reaction may be total RNA, 20 mRNA, and/or cDNA obtained by reverse transcription of mRNA prepared from, for example, human or non-human cell lines or tissue, such as prostate, rectum, colon, or adrenal gland, known or suspected to express an allele of a NHP gene. The PCR product can be subcloned and sequenced to ensure that the 25 amplified sequences represent the sequence of the desired NHP gene. The PCR fragment can then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment can be labeled and used to screen a cDNA library, such as a bacteriophage cDNA library. Alternatively, the labeled fragment 30 can be used to isolate genomic clones via the screening of a genomic library.

PCR technology can also be used to isolate full length cDNA sequences. For example, RNA can be isolated, following standard

procedures, from an appropriate cellular or tissue source (i.e., one known, or suspected, to express a NHP gene, such as, for example, testis tissue). A reverse transcription (RT) reaction can be performed on the RNA using an oligonucleotide primer

5 specific for the most 5' end of the amplified fragment for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" using a standard terminal transferase reaction, the hybrid may be digested with RNase H, and second strand synthesis may then be primed with a complementary primer.

10 Thus, cDNA sequences upstream of the amplified fragment can be isolated. For a review of cloning strategies that can be used, see e.g., Sambrook *et al.*, 1989, *supra*.

A cDNA encoding a mutant NHP gene can be isolated, for example, by using PCR. In this case, the first cDNA strand may be

15 synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated from tissue known or suspected to be expressed in an individual putatively carrying a mutant NHP allele, and by extending the new strand with reverse transcriptase. The second strand of the cDNA is then synthesized using an oligonucleotide

20 that hybridizes specifically to the 5' end of the normal gene. Using these two primers, the product is then amplified via PCR, optionally cloned into a suitable vector, and subjected to DNA sequence analysis through methods well known to those of skill in the art. By comparing the DNA sequence of the mutant NHP allele

25 to that of a corresponding normal NHP allele, the mutation(s) responsible for the loss or alteration of function of the mutant NHP gene product can be ascertained.

Alternatively, a genomic library can be constructed using DNA obtained from an individual suspected of or known to carry a

30 mutant NHP allele (e.g., a person manifesting a NHP-associated phenotype such as, for example, obesity, high blood pressure, etc.), or a cDNA library can be constructed using RNA from a tissue known, or suspected, to express a mutant NHP allele. A

normal NHP gene, or any suitable fragment thereof, can then be labeled and used as a probe to identify the corresponding mutant NHP allele in such libraries. Clones containing mutant NHP gene sequences can then be purified and subjected to sequence analysis 5 according to methods well known to those skilled in the art.

Additionally, an expression library can be constructed utilizing cDNA synthesized from, for example, RNA isolated from a tissue known, or suspected, to express a mutant NHP allele in an individual suspected of or known to carry such a mutant allele.

10 In this manner, gene products made by the putatively mutant tissue may be expressed and screened using standard antibody screening techniques in conjunction with antibodies raised against a normal NHP product, as described below. (For screening techniques, see, for example, Harlow, E. and Lane, eds., 1988, "Antibodies: A 15 Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor.)

Additionally, screening can be accomplished by screening with labeled NHP fusion proteins, such as, for example, AP-NHP or NHP-AP fusion proteins. In cases where a NHP mutation results in an 20 expressed gene product with altered function (e.g., as a result of a missense or a frameshift mutation), polyclonal antibodies to a NHP are likely to cross-react with a corresponding mutant NHP gene product. Library clones detected via their reaction with such labeled antibodies can be purified and subjected to sequence 25 analysis according to methods well known in the art.

The invention also encompasses (a) DNA vectors that contain any of the foregoing NHP coding sequences and/or their complements (i.e., antisense); (b) DNA expression vectors that contain any of the foregoing NHP coding sequences operatively associated with a 30 regulatory element that directs the expression of the coding sequences (for example, baculo virus as described in U.S. Patent No. 5,869,336 herein incorporated by reference); (c) genetically engineered host cells that contain any of the foregoing NHP coding

sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell; and (d) genetically engineered host cells that express an endogenous NHP gene under the control of an exogenously introduced 5 regulatory element (i.e., gene activation). As used herein, regulatory elements include but are not limited to inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression. Such regulatory elements include but are not limited 10 to the cytomegalovirus hCMV immediate early gene, regulatable, viral (particularly retroviral LTR promoters) the early or late promoters of SV40 adenovirus, the *lac* system, the *trp* system, the *TAC* system, the *TRC* system, the major operator and promoter regions of phage lambda, the control regions of fd coat protein, 15 the promoter for 3-phosphoglycerate kinase (PGK), the promoters of acid phosphatase, and the promoters of the yeast α -mating factors.

The present invention also encompasses antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists and agonists of the NHP, as well as compounds or nucleotide constructs 20 that inhibit expression of a NHP gene (transcription factor inhibitors, antisense and ribozyme molecules, or gene or regulatory sequence replacement constructs), or promote the expression of a NHP (e.g., expression constructs in which NHP coding sequences are operatively associated with expression 25 control elements such as promoters, promoter/enhancers, etc.).

The NHPs or NHP peptides, NHP fusion proteins, NHP nucleotide sequences, antibodies, antagonists and agonists can be useful for the detection of mutant NHPs or inappropriately expressed NHPs for the diagnosis of disease. The NHP proteins or peptides, NHP 30 fusion proteins, NHP nucleotide sequences, host cell expression systems, antibodies, antagonists, agonists and genetically engineered cells and animals can be used for screening for drugs (or high throughput screening of combinatorial libraries)

effective in the treatment of the symptomatic or phenotypic manifestations of perturbing the normal function of NHP in the body. The use of engineered host cells and/or animals may offer an advantage in that such systems allow not only for the 5 identification of compounds that bind to the endogenous receptor for an NHP, but can also identify compounds that trigger NHP-mediated signal transduction.

Finally, the NHP products can be used as therapeutics. For example, soluble derivatives such as NHP peptides/domains 10 corresponding the NHPs, NHP fusion protein products (especially NHP-Ig fusion proteins, *i.e.*, fusions of a NHP, or a domain of a NHP, to an IgFc), NHP antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists or agonists (including compounds that modulate signal transduction which may act on 15 downstream targets in a NHP-mediated signal transduction pathway) can be used to directly treat diseases or disorders. For instance, the administration of an effective amount of soluble NHP, or a NHP-IgFc fusion protein or an anti-idiotypic antibody (or its Fab) that mimics the NHP could activate or effectively 20 antagonize the endogenous NHP receptor. Nucleotide constructs encoding such NHP products can be used to genetically engineer host cells to express such products *in vivo*; these genetically engineered cells function as "bioreactors" in the body delivering a continuous supply of a NHP, a NHP peptide, or a NHP fusion 25 protein to the body. Nucleotide constructs encoding functional NHPs, mutant NHPs, as well as antisense and ribozyme molecules can also be used in "gene therapy" approaches for the modulation of NHP expression. Thus, the invention also encompasses pharmaceutical formulations and methods for treating biological 30 disorders.

A knockout ES cell clone has been produced in a murine gene encoding an ortholog of the disclosed NHPs.

Various aspects of the invention are described in greater detail in the subsections below.

5.1 THE NHP SEQUENCES

5 The cDNA sequences (SEQ ID NOS: 1 and 3) and the corresponding deduced amino acid sequence (SEQ ID NO: 2) of the described NHPs are presented in the Sequence Listing. The NHP genes were obtained from human testis and placenta cDNA libraries using probes and/or primers generated from human gene trapped 10 sequence tags. Expression analysis has provided evidence that the described NHPs can be expressed, for example, in human testis, prostate, and gene trapped human cells. In addition to the genes encoding trypsin inhibitors, the described NHPs share significant 15 similarity to a variety of cancer pathogenesis proteins, sperm glycoproteins, and secretory proteins.

The described open reading frames can also contain several polymorphisms including an C to T transition corresponding to base 81 of SEQ ID NO:1, a G to C transversion corresponding to base 965 of SEQ ID NO:1 (changing a serine to a threonine), and a C to G 20 transversion corresponding to base 165 of the 5' UTR of SEQ ID NO:3. SEQ ID NO:3 describes a full length ORF with flanking 5' and 3' sequences.

5.2 NHPS AND NHP POLYPEPTIDES

25 NHPs, polypeptides, peptide fragments, mutated, truncated, or deleted forms of the NHPs, and/or NHP fusion proteins can be prepared for a variety of uses. These uses include but are not limited to the generation of antibodies, as reagents in diagnostic assays, the identification of other cellular gene products related 30 to a NHP, as reagents in assays for screening for compounds that can be used as pharmaceutical reagents useful in the therapeutic treatment of mental, biological, or medical disorders and disease.

The Sequence Listing discloses the amino acid sequences encoded by the described NHP genes. The NHPs have initiator methionines in DNA sequence contexts consistent with a translation initiation site, and further incorporate a hydrophobic leader 5 sequence characteristic of secreted proteins.

The NHP amino acid sequences of the invention include the amino acid sequence presented in the Sequence Listing as well as analogues and derivatives thereof. Further, corresponding NHP homologues from other species are encompassed by the invention. 10 In fact, any NHP protein encoded by the NHP nucleotide sequences described above are within the scope of the invention, as are any novel polynucleotide sequences encoding all or any novel portion of an amino acid sequence presented in the Sequence Listing. The degenerate nature of the genetic code is well known, and, 15 accordingly, each amino acid presented in the Sequence Listing, is generically representative of the well known nucleic acid "triplet" codon, or in many cases codons, that can encode the amino acid. As such, as contemplated herein, the amino acid sequences presented in the Sequence Listing, when taken together 20 with the genetic code (see, for example, Table 4-1 at page 109 of "Molecular Cell Biology", 1986, J. Darnell et al. eds., Scientific American Books, New York, NY, herein incorporated by reference) are generically representative of all the various permutations and combinations of nucleic acid sequences that can encode such amino 25 acid sequences.

The invention also encompasses proteins that are functionally equivalent to the NHPs encoded by the presently described nucleotide sequences as judged by any of a number of criteria, including, but not limited to, the ability to bind and cleave a 30 substrate of a NHP, or the ability to effect an identical or complementary downstream signal transduction pathway, or a change in cellular metabolism (e.g., proteolytic activity, ion flux, tyrosine phosphorylation, etc.). Such functionally equivalent NHP

proteins include, but are not limited to, additions or substitutions of amino acid residues within the amino acid sequence encoded by the NHP nucleotide sequences described above, but which result in a silent change, thus producing a functionally equivalent gene product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

A variety of host-expression vector systems can be used to express the NHP nucleotide sequences of the invention. Where, as in the present instance, the NHP peptide or polypeptide is thought to be a soluble or secreted molecule, the peptide or polypeptide can be recovered from the culture media. Such expression systems also encompass engineered host cells that express a NHP, or functional equivalent, *in situ*, i.e. anchored to the cell membrane. Purification or enrichment of a NHP from such expression systems can be accomplished using appropriate detergents and lipid micelles and methods well known to those skilled in the art. Alternatively, such engineered host cells themselves may be used in situations where it is important not only to retain the structural and functional characteristics of the NHP, but to assess biological activity, e.g., in drug screening assays.

The expression systems that may be used for purposes of the invention include but are not limited to microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors

containing NHP nucleotide sequences; yeast (e.g., *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing NHP nucleotide sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus)

5 containing NHP sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing NHP nucleotide sequences; or mammalian cell systems 10 (e.g., COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

15 In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the NHP product being expressed. For example, when a large quantity of such a protein is to be produced for the generation of pharmaceutical compositions of or containing NHP, or for raising 20 antibodies to a NHP, vectors that direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which a NHP coding sequence may be ligated 25 individually into the vector in frame with the *lacZ* coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion 30 proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by

elution in the presence of free glutathione. The PGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

5 In an insect system, *Autographa californica* nuclear polyhidrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. A NHP gene coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed 10 under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of NHP gene coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These 15 recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed (e.g., see Smith et al., 1983, *J. Virol.* 46: 584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as 20 an expression vector, the NHP nucleotide sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non- 25 essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing a NHP product in infected hosts (e.g., See Logan & Shenk, 1984, *Proc. Natl. Acad. Sci. USA* 81:3655-3659). Specific initiation signals may also be required for efficient translation 30 of inserted NHP nucleotide sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire NHP gene or cDNA, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression

vector, no additional translational control signals may be needed. However, in cases where only a portion of a NHP coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, 5 the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the 10 inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (See Bittner et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and 15 processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and 20 modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, 25 glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, human cell lines.

For long-term, high-yield production of recombinant proteins, 30 stable expression is preferred. For example, cell lines which stably express the NHP sequences described above can be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with

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DNA controlled by appropriate expression control elements (e.g., promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be 5 allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into 10 cell lines. This method may advantageously be used to engineer cell lines which express the NHP product. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the NHP product.

A number of selection systems may be used, including but not 15 limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes 20 can be employed in tk⁻, hgprt⁻ or aprt⁻ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, 25 which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147).

30 Alternatively, any fusion protein can be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al.

allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, et al., 1991, Proc. Natl. Acad. Sci. USA 88:8972-8976). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such 5 that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni^{2+} ·nitrioloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

10

5.3 ANTIBODIES TO NHP PRODUCTS

Antibodies that specifically recognize one or more epitopes of a NHP, or epitopes of conserved variants of a NHP, or peptide fragments of a NHP are also encompassed by the invention. Such 15 antibodies include but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, $\text{F}(\text{ab}')_2$ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the 20 above.

The antibodies of the invention may be used, for example, in the detection of NHP in a biological sample and may, therefore, be utilized as part of a diagnostic or prognostic technique whereby patients may be tested for abnormal amounts of NHP. Such 25 antibodies may also be utilized in conjunction with, for example, compound screening schemes, as described, below, in Section 5.5, for the evaluation of the effect of test compounds on expression and/or activity of a NHP gene product. Additionally, such antibodies can be used in conjunction gene therapy to, for 30 example, evaluate the normal and/or engineered NHP-expressing cells prior to their introduction into the patient. Such antibodies may additionally be used as a method for the inhibition

of abnormal NHP activity. Thus, such antibodies may, therefore, be utilized as part of treatment methods.

For the production of antibodies, various host animals may be immunized by injection with the NHP, an NHP peptide (e.g., one corresponding to a functional domain of an NHP), truncated NHP polypeptides (NHP in which one or more domains have been deleted), functional equivalents of the NHP or mutated variant of the NHP. Such host animals may include but are not limited to pigs, rabbits, mice, goats, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's adjuvant (complete and incomplete), mineral salts such as aluminum hydroxide or aluminum phosphate, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Alternatively, the immune response could be enhanced by combination and/or coupling with molecules such as keyhole limpet hemocyanin, tetanus toxoid, diphtheria toxoid, ovalbumin, cholera toxoid or fragments thereof. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, can be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein, (1975, *Nature* 256:495-497; and U.S. Patent No. 4,376,110), the human B-cell hybridoma technique (Kosbor *et al.*, 1983, *Immunology Today* 4:72; Cole *et al.*, 1983, *Proc. Natl. Acad. Sci. USA* 80:2026-2030), and the EBV-hybridoma technique (Cole *et al.*, 1985, *Monoclonal*

Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be 5 cultivated in vitro or in vivo. Production of high titers of mAbs in vivo makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison *et al.*, 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger *et al.*, 1984, Nature, 10 312:604-608; Takeda *et al.*, 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity 15 can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region. Such technologies are described in U.S. Patents Nos. 6,075,181 20 and 5,877,397 and their respective disclosures which are herein incorporated by reference in their entirety.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; Bird, 1988, Science 242:423-426; Huston *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward *et al.*, 1989, Nature 25 334:544-546) can be adapted to produce single chain antibodies against NHP gene products. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

30 Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include, but are not limited to: the F(ab')₂ fragments which can be produced by pepsin digestion of the antibody molecule and the

Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed (Huse *et al.*, 1989, *Science*, 246:1275-1281) to allow rapid and easy identification of 5 monoclonal Fab fragments with the desired specificity.

Antibodies to a NHP can, in turn, be utilized to generate anti-idiotype antibodies that "mimic" a given NHP, using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, 1993, *FASEB J* 7(5):437-444; and Nissinoff, 1991, 10 *J. Immunol.* 147(8):2429-2438). For example antibodies which bind to a NHP domain and competitively inhibit the binding of NHP to its cognate receptor can be used to generate anti-idiotypes that "mimic" the NHP and, therefore, bind and activate or neutralize a receptor. Such anti-idiotypic antibodies or fragments of such 15 anti-idiotypes can be used in therapeutic regimens involving a NHP signaling pathway.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and 20 functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the 25 scope of the appended claims.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule comprising at least 24 contiguous bases of nucleotide sequence first disclosed
5 in the NHP gene described in SEQ ID NO: 1.

2. An isolated nucleic acid molecule comprising a nucleotide sequence that:

(a) encodes the amino acid sequence shown in SEQ ID
10 NO: 2; and
(b) hybridizes under stringent conditions to the nucleotide sequence of SEQ ID NO: 1 or the complement thereof.

15 3. An expression vector comprising a polynucleotide sequence encoding the amino acid sequence shown in SEQ ID NO:2.

ABSTRACT

Novel human polynucleotide and polypeptide sequences are disclosed that can be used in therapeutic, diagnostic, and pharmacogenomic applications.

PATENT APPLICATION

ATTORNEY DOCKET NO. LEX-0042-USA

**DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION**

As a below named inventor, I hereby declare that:

My residence/post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Novel Human Protease Inhibitor-Like Proteins and Polynucleotides Encoding the Same

the specification of which is attached hereto unless the following box is checked:

() was filed on _____ as US Application Serial No. or PCT International Application

Number _____ and was amended on _____ (if applicable).

I hereby state that I have reviewed and understood the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose all information which is material to patentability as defined in 37 CFR 1.56.

Foreign Application(s) and/or Claim of Foreign Priority

I hereby claim foreign priority benefits under Title 35, United States Code Section 119 of any foreign application(s) for patent or inventor(s) certificate listed below and have also identified below any foreign application for patent or inventor(s) certificate having a filing date before that of the application on which priority is claimed:

COUNTRY	APPLICATION NUMBER	DATE FILED	PRIORITY CLAIMED UNDER 35 U.S.C. 119
			YES: <u> </u> NO: <u> </u>
			YES: <u> </u> NO: <u> </u>

Provisional Application

I hereby claim the benefit under Title 35, United States Code Section 119(e) of any United States provisional application(s) listed below:

APPLICATION SERIAL NUMBER	FILING DATE
60/156,101	09/24/1999

U.S. Priority Claim

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NUMBER	FILING DATE	STATUS(patented/pending/abandoned)

POWER OF ATTORNEY:

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) listed below to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION (continued)

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